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Chaperones, protein homeostasis & protein aggregation diseases

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CHAPTER 5

HSPA1A-independent suppression of PARK2 C289G protein aggregation by human small heat shock proteins.

Melania Minoia, Corien Grit, Harm H. Kampinga

Manuscript submitted

Abstract

The C289G mutation of the parkin E3-ubiquitin protein ligase (PARK2) is associated with autosomal recessive juvenile onset Parkinson's disease and was found to be associated with protein aggregation. Members of the human small heat shock proteins (HSPBs) have been implicated in protein degradation and prevention of protein aggregation. In this study, we show that of the ten HSPB members, individual overexpression of HSPB1, HSPB2, HSPB4 and HSPB7 suppresses PARK2 C289G-associated protein aggregation. Intriguingly, the protective actions of these HSPBs are not impaired upon inactivation of the ATP-dependent HSP70 chaperone machines. Depending on the HSPB member the protective actions involve either autophagic or proteasomal degradation pathways.

Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is characterized by degeneration of dopaminergic neurons and the presence of cytoplasmic inclusions, called Lewy bodies, in the substantia nigra. PD is mostly sporadic, however several familial forms are known, including mutations in the PARK2 gene. PARK2 codes for the parkin RBR E3-ubiquitin protein ligase, which has been associated with autosomal recessive juvenile onset Parkinson's disease (ARJPD) [1]. PARK2/PARKIN is an E3-ubiquitin protein ligase, predominantly expressed in the muscles and the brain [1,2]. PARK2 consists of an ubiquitin-like (UBL) domain at the N-terminus and two RING-domains at the C-terminus, of which the latter are essential for PARK2's ubiquitin ligase function [2,3]. Besides its ubiquitin ligase function, PARK2 is a key player in the mitochondrial quality control system [4]. One of the recessive mutations in PARK2 is the PARK2 C289G mutation, located at the RING1 domain and suggests loss of function as the prevalent cause in juvenile onset PD. Yet, expression of PARK2 C289G is associated with its sequestration into protein aggregates, as the affected cysteine residue is important for the structural stability of the PARK2 protein [5–7], which suggest that this PARK2 mutant may (also) exert dominant negative effects or have a gained toxic (aggregation-related) function. In line, Khan *et al.* (2003) reported on twenty-four ARJPD cases of which ten patients had only one allele that was mutated [8]. In addition, non-PD patients with a single PARK2 mutant allele were found to manifest behavioural disorders and nigrostriatal dysfunction [9,10].

A potential strategy to counteract the protein aggregation associated with PARK2 C289G expression may be achieved by the action of members of the family of small heat shock proteins (HSPBs). All HSPBs contain an α -crystallin domain, flanked by variable C- and N-terminal regions [11,12]. For several of the HSPB members, it was shown that they can bind to non-native proteins and either facilitate their correct folding or assist in their degradation [11,13]. The HSPBs lack ATPase activity and therefore are generally thought to require the ATP-dependent HSP70 chaperone machinery for their activities [13], although HSP70-independent chaperone actions have also been suggested [12,14]. The importance of HSPBs in neuro/muscular functioning appears from the many muscular and neurodegenerative diseases caused by mutations in the HSPBs members [11,12,15] and their up-regulation in numerous pathological conditions [16,17]. Moreover, up-regulation of several HSPBs was found to be protective in a number of protein aggregation disease models for polyglutamine (polyQ) diseases and Amyotrophic Lateral Sclerosis (ALS) [11,14,15,18,19]. These data prompted us to test if members of the HSPB family might also be able to suppress aggregation related to expression of the PARK2 C289G mutant. Our dedicated screen revealed that HSPB1, HSPB2, HSPB4 and HSPB7 are potent suppressors of PARK2 C289G aggregation in mammalian cells. This result differs from what we found for polyQ diseases, where HSPB7, HSPB8 and HSPB9 were the

most protective members [14,20]. The anti-aggregation activity of the HSPBs was not found to depend on the Hsp70/HSPA chaperone system, but either involved support of clearance of PARK2 C289G aggregates via autophagy (HSPB7) or via proteasomal degradation of PARK2 C289G (HSPB1).

Results

HSPB1, HSPB2, HSPB4 and HSPB7 are able to suppress PARK2 C289G aggregation.

The C289G missense mutation in the PARK2 RING-domain alters the intracellular distribution pattern of PARK2 and decreases its solubility [5,21]. We confirmed that in HEK293 cells ectopically expressed wild type (WT) PARK2 is diffusely distributed, whereas PARK2 C289G forms inclusions in 56.3% of the cells within twenty-four hours (Fig. 1 A). In line, the majority of PARK2 wild type (50 kDa) was found in the Triton X100-soluble fraction, whereas a major fraction of the PARK2 mutant had become TX-100 insoluble (Fig. 1 B). The appearance of a smear above the 50kDa band of PARK2 mutant has previously shown to be attributed to the loss of inhibition of its auto-ubiquitination activity [7]. The lower 42 kDa band is an N-terminal truncated PARK2 species resulting from an internal initiation site within the PARK2 gene [22]. In addition, a significant amount of PARK2 mutant was found in the stacking gel (high molecular weight, HMW: Fig. 1 B), implying formation of SDS-insoluble aggregates.

HSPBs have been shown to prevent the aggregation of several aggregation-inducing proteins, their effectiveness seemingly depending on the substrate and physical properties of the aggregate it forms [11]. When co-expressing the different HSPB members with PARK2 C289G (Fig. S1 A), a major reduction in the aggregation of PARK2 C289G was seen in cells co-expressing HSPB1, HSPB2, HSPB4 or HSPB7, but not in cells co-expressing the other HSPB members (Fig. 1 C). Immunocytochemistry confirmed these effects, showing significantly lower percentages of PARK2 puncta in cells co-expressing HSPB1, HSPB2, HSPB4 or HSPB7 compared to control conditions (Fig. 1 D, E). Expression of HSPB6, which did not affect PARK2 insolubilization (Fig. 1 C), also did not effect puncta formation of PARK2 C289G (Fig. 1 E). Categorizing PARK2 C289G-aggregates in abundance and size (Fig. S1 B) reveals that all effective HSPB members especially reduced the number of cells with large(r) aggregates (Fig. 1 E). Confocal analysis furthermore revealed that HSPB2, HSPB4 and HSPB7 co-localize with PARK2 aggregates, whereas HSPB1 does not (Fig. 2 A, arrow). Interestingly, HSPB2 and HSPB4 were found inside the aggregates, whereas HSPB7 appears to be more at the rim of the aggregates, similar as was found before for polyQ aggregates [14]. Therefore, although the four HSPBs all suppressed PARK2 insolubilization, their mode of action may actually differ, especially that of HSPB1.

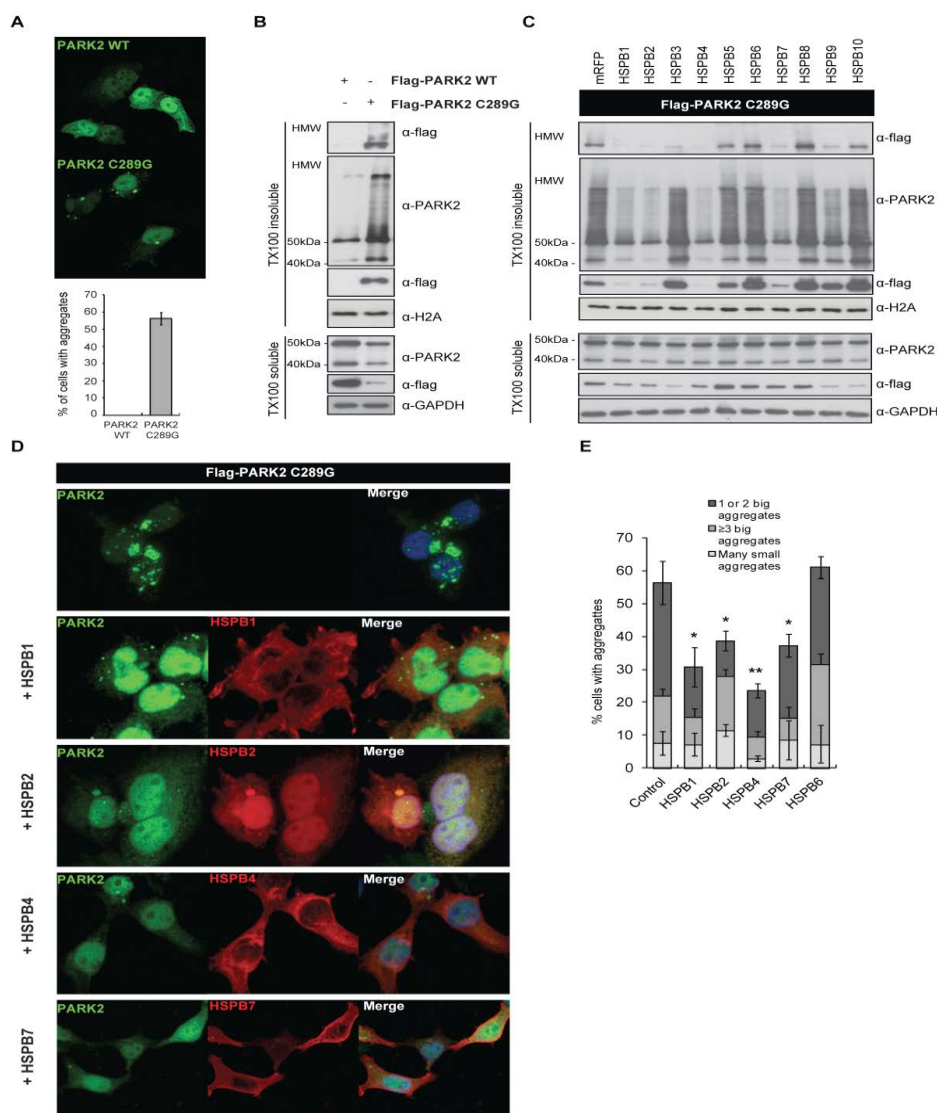


Figure 1. Suppression of PARK2 C289G aggregation by HSPBs.

(A) HEK-293 cells were transfected for 24hr with Flag-tagged PARK2 WT or PARK2 C289G. Subcellular distribution of PARK2 (green) was investigated by immunofluorescence. Diagram shows percentage of PARK2-aggregates. (B) Cells, transfected as in (A), were fractionated in TX-100 soluble and insoluble proteins and analyzed by western blot. (C) HEK-293 cells were transfected for 24hr with Flag-tagged PARK2 C289G alone or together with each of the different HSPB proteins. Cells were fractionated in TX-100 soluble and insoluble proteins and analyzed by western blot. HSPB expression levels are shown in Fig. E1A (D) Immunofluorescent staining of Flag-tagged PARK2 (green), HSPBs (red) and dapi (blue) in HEK293 cells, transfected as in (C). (E) Diagram shows percentage of flag-tagged PARK2 expressing cells with aggregates (*= $p < 0.05$; **= $p < 0.001$; $n > 3$ independent samples, mean \pm SEM). Cells were divided in different categories as shown in Fig. E1 B. HSPB6 was added as a negative control. HMW: High molecular weight.

The Hsp70/HSPA chaperone machinery is not required for the HSPBs anti-aggregation activity.

In vitro studies have shown that many HSPBs can prevent irreversible aggregation of clients directly and without need of additional activities. However, the HSPBs generally are thought to require cooperation with ATP-dependent chaperones, like the Hsp70/HSPA machines, for client release and subsequent processing [23–26]. To investigate whether the effects of HSPB1, HSPB2, HSPB4 and HSPB7 on PARKIN C289G aggregation depend on a collaboration with Hsp70/HSPA members, we first down-regulated the expression of HSPA1A to below 20% by siRNA (Fig. 3 A), followed by treatment with the Hsp70/HSPA inhibitor VER-155008 (Fig. 3 A). HSPA1A depleted cells showed increased PARK2 C289G aggregation (Fig. 3 A), confirming a protective role of endogenous HSPA1A in PARK2 C289G aggregation. However, despite the HSPA1A depletion, the four HSPBs were still able to suppress PARK2 mutant aggregation, even though HSPA1A depletion had led to an higher burden of aggregation-prone PARK2 C289G. Next, we inhibited Hsp70 folding capacity using VER-155008, a potent inhibitor of the ATP turnover of Hsp70/HSPA family [27]. Treatment of cells with VER-155008 as such further enhanced PARKIN C289G aggregation (Fig. 3 B), confirming the siRNA data. Furthermore, this shows that endogenously expressed HSP70 family members already provide aggregation protection and that the drug was effective at the conditions used here. Yet, like with the knockdown of HSPA1A, the VER-155008 treatment did not abolish the protective effects of HSPB1, HSPB2, and HSPB4 on PARK2 C289G aggregation, even though VER-155008 has led to an increased burden of aggregation-prone PARK2 C289G (Fig. 3 B). Together, these results suggest that these HSPBs are able to suppress PARK2 C289G aggregation in an Hsp70/HSPA-independent manner.

HSPBs use the proteasome system or the autophagy pathway to degrade mutated PARK2.

HSPB proteins have been repeatedly associated with enhanced protein degradation through either the ubiquitin–proteasome system [28–30], or through autophagy [14,20]. To test whether HSPB1, HSPB2, HSPB4 and HSPB7 depend on a functional autophagosomal machine to exert their protective effects on PARK2 C289G aggregation, we inhibited autophagy with 3-methyladenine (3-MA) and Wortmannin. The inhibition of autophagy enhanced PARK2 C289G aggregation (Fig. S2), suggesting that constitutive autophagy is involved in the normal clearance of misfolded PARK2, as has been suggested before [31]. Under such conditions HSPB1, HSPB2 and HSPB4 were still able to prevent PARK2 aggregate formation. However, the protective action of HSPB7 was strongly reduced (Fig. 4 A). To further test whether HSPB7 indeed inhibits PARK2 C289G aggregation using autophagy, we tested the capacity of HSPB7 to suppress PARK2 C289G aggregation in autophagy

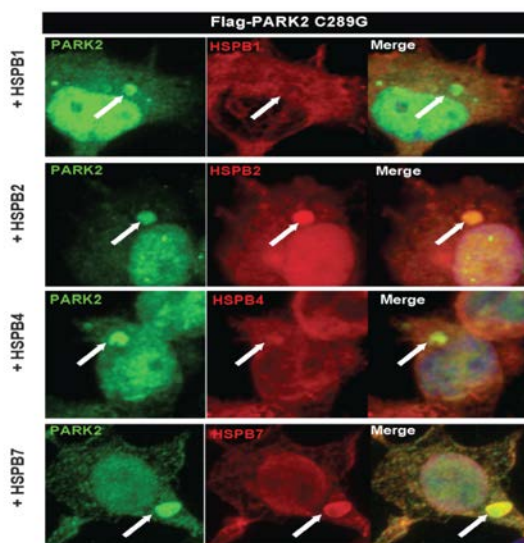


Figure 2. Co-localization of the HSPBs with PARK2 C289G aggregates.

(A) Immunofluorescent staining of Flag-tagged PARK2 (green), HSPBs (red) and dapi (blue) in HEK293 cells, as transfected in Fig. 1D.

deficient (ATG5 $-/-$) murine embryonic fibroblasts (MEFs). In these cells, HSPB1, HSPB2 and HSPB4 still led to a reduction of PARK2 C289G aggregates (Fig. 4 B), consistent with their action being autophagy un-related. However, HSPB7 activity on PARK2 C289G aggregates was abrogated in ATG5 $-/-$ cells (Fig. 4 B). These results are in line with our previous findings on polyQ proteins, in which HSPB7 was found to prevent polyQ aggregation in an autophagy dependent manner [14].

To test whether HSPB1, HSPB2 and HSPB4 might suppress PARK2 C289G aggregation via the ubiquitin–proteasome system, we analysed their anti-aggregation effects upon proteasomal inhibition. Treatment with Bortezomib successfully inhibited the proteasome as evidenced by the accumulation of ubiquitinated proteins (Fig. 4 A). Surprisingly, treatment with Bortezomib alone (Fig. S2) or in combination with the four HSPBs (Fig. 4 A) reduced the level of soluble PARK2. It has been recently shown that Bortezomib treatment leads to activation of autophagy [32,33], hence we conclude from these data that mutant PARK2 C289G may be primarily degraded through autophagy under such conditions. Yet, Bortezomib treatment significantly (albeit not completely) reduced the protective effects of HSPB1 and HSPB4 on PARK2 C289G aggregation, whilst having only a modest effect on HSPB7 mediated protection and no effect on HSPB2-mediated protection (Fig. 4 A). These data suggest that HSPB1 and HSPB4 anti-aggregation activities, at least partially, require an active proteasome. In conclusion, the different protective HSPBs prevent PARK2 aggregation via mechanistically distinct pathways.

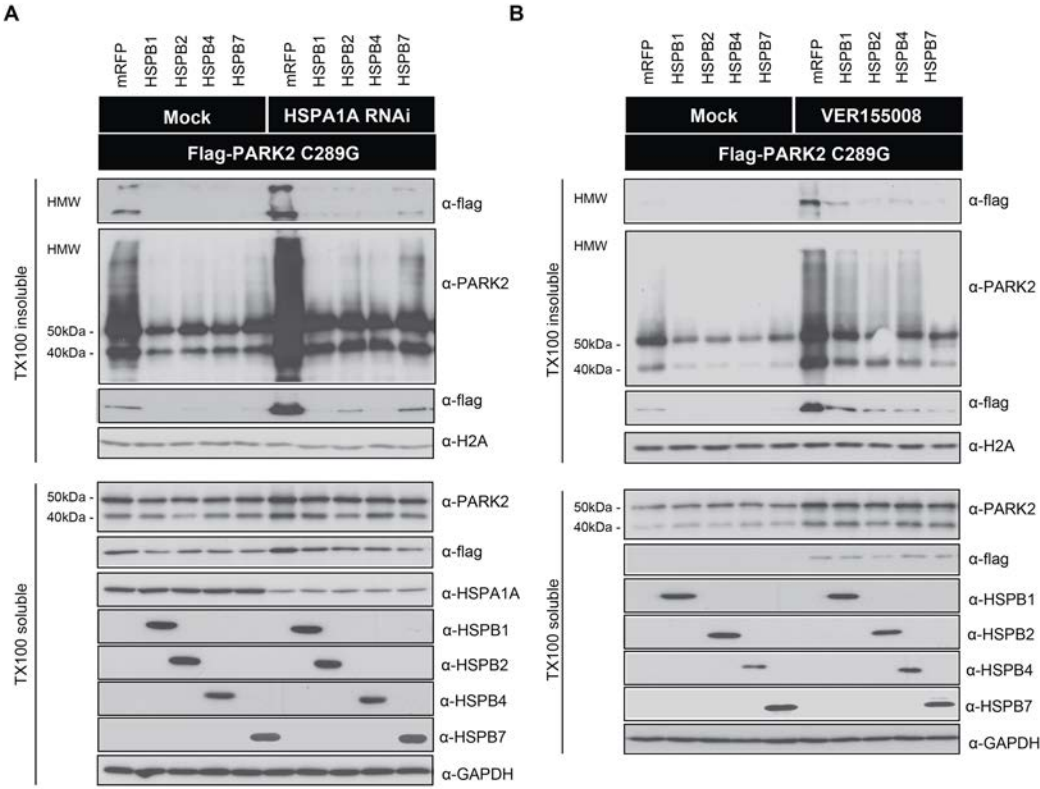


Figure 3. HSPB1, HSPB2, HSPB4 and HSPB7 suppress PARK2 C289G aggregation in a HSPA1A-independent manner.

(A) HEK-293 cells were transfected with nonsense or HSPA1A RNAi. 48hr post-transfection cells were transfected with Flag-tagged PARK2 C289G and mRFP-, HSPB1-, HSPB2-, HSPB4- or HSPB7-encoding vectors and fractionated 24 hours later in TX-100 soluble and insoluble proteins. HMW: High molecular weight. (B) HEK-293 cells were transfected for 24hr with Flag-tagged PARK2 C289G and mRFP-, HSPB1-, HSPB2-, HSPB4- or HSPB7-encoding vector. Cells were treated with VER-155008 (40 mM, 24hr) and fractionated in TX-100 soluble and insoluble proteins.

Discussion

In this manuscript we show that HSPB1, HSPB2, HSPB4 and HSPB7 can protect cells from C289G PARK2-aggregation in an Hsp70/HSPA-independent manner and via different degradation pathways.

The ability of the several HSPB family members to protect against protein aggregation seems to differ substantially. Up to now, HSPB1, HSPB5 and HSPB8 are the most widely studied members. They have been found to reduce protein aggregates caused by proteins containing an expanded polyQ tract (HSPB8: [20]), tau (HSPB1:

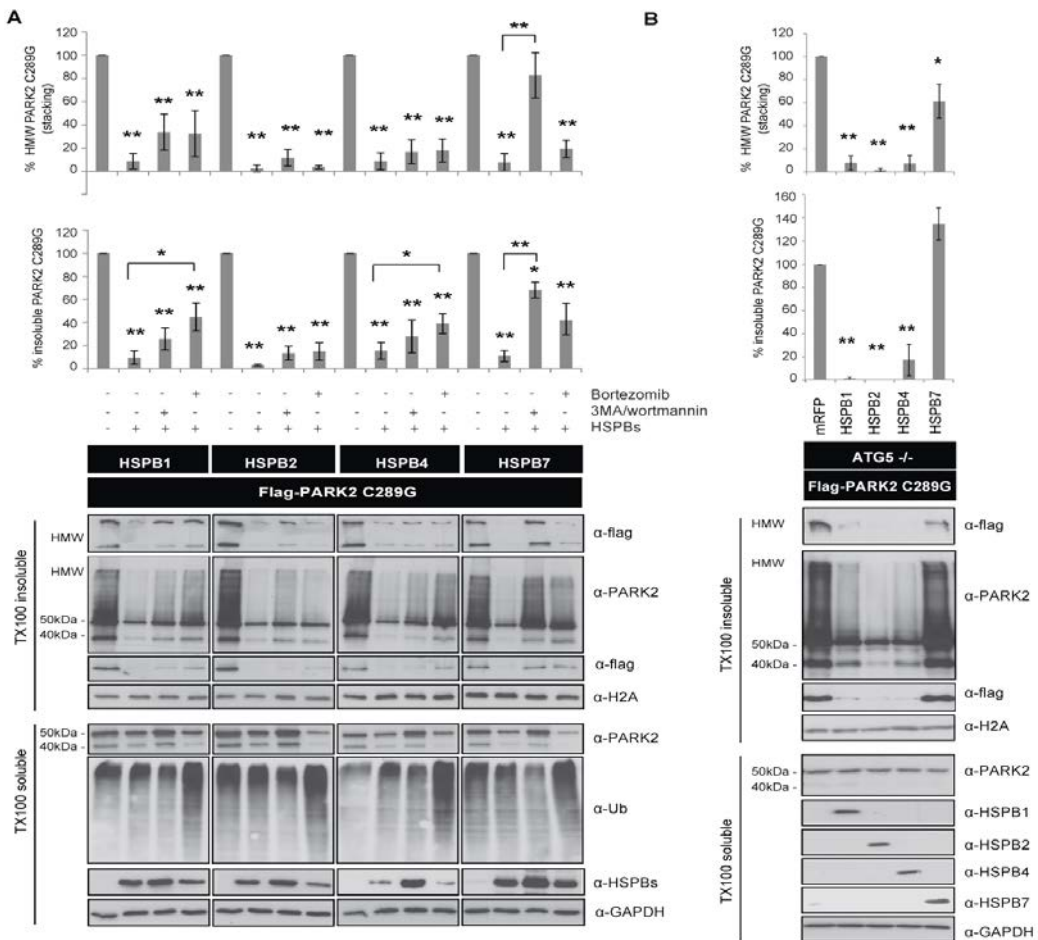


Figure 4. Role of ubiquitin proteasome system or the autophagy pathway in HSPB effects on PARK2 C289G aggregates.

(A) HEK293 cells, transfected as in Fig. 3 A, were treated with Bortezomib (100 nM) or with 3-MA (20mM) and Wortmannin (200 nM) overnight. Cells were fractionated in TX-100 and analyzed for PARK2 C289G aggregates (*= $p < 0.05$; **= $p < 0.001$; $n > 3$ independent samples, mean \pm SEM).. (B) ATG5^{-/-} MEF cells were transfected for 24hr with Flag-tagged PARK2 C289G and mRFP-, HSPB1-, HSPB2-, HSPB4- or HSPB7-encoding vector and analyzed for PARK2 C289G aggregates (*= $p < 0.05$; **= $p < 0.001$; $n > 3$ independent samples, mean \pm SEM). HMW: High molecular weight.

[34–36]), amyloid-beta (HSPB1, HSPB5 and HSPB8: [37,38]), TDP43 (HSPB8: [15]) and SOD1 (HSPB1, HSPB5 and HSPB8: [18,39]). However, only little is known about the protective effects of the other HSPBs. In the last years, a few comparative studies have been done in which the ten HSPB members were directly compared for their activity towards various substrates within the same cell models systems. Comparing the ten HSPB members for canonical refolding activity, using the firefly

luciferase as a model substrate, revealed that only HSPB1, HSPB4 and HSPB5 up-regulation lead to improved refolding after heat shock [14,40]. In the same HEK293 cells, up-regulation of especially HSPB7, but also HSPB6, HSPB8 and HSPB9 was found to be effective in preventing polyQ-proteins aggregation (mutant huntingtin and ataxin-3) [14,20], whereas HSPB8 was the only HSPB able to prevent aggregation of mutant TDP43 (associated with ALS) [15]. In cardiomyocytes, upregulation of especially HSPB1, but also HSPB6, HSPB7, HSPB8 was effective in protecting cytoskeletal elements from tachypacing [41]. Here, we show again, using HEK293 cells, that HSPB1, HSPB2 and HSPB4 as well as HSPB7 suppress aggregation of PARK2 C298G mutant (Fig. 1). These data clearly indicate that the different human HSPB members display substrate specificity, albeit with some overlap [11].

In addition to substrate specificity, the mechanisms of action of the different HSPB members seem to differ drastically. Cell-free studies have shown that some HSPB members prevent substrate aggregation, hereby keeping them competent for HSP70-dependent refolding [24–26]. In cells, handling of heat-denatured luciferase by some HSP members also was found to be dependent on a functional Hsp70 machinery [14,40]. Besides supporting (re)folding, HSPBs substrate holding in cells might also assist in proper client disposal. In our current report, such seems to be the case for HSPB1 and HSPB4 that require a functional proteasomal degradation pathway to prevent PARK C298G aggregation (Fig. 4). Interestingly, however, this effect was independent of the Hsp70/HSPA chaperone machinery (Fig. 3). It is tempting to speculate that the proteasome capping proteins might be able to release clients from HSPB1 directly, without the need of the Hsp70/HSPA machine. In support of such a hypothesis, HSPB1 was found to co-localize with both ubiquitinated proteins and proteasomal inclusion bodies [28] and HSPB1 was found to directly interact with the proteasome [30,42]. Such a mode of action would also explain why HSPB1 is not located with the aggregates (Fig. 2) as such an action would be prior to aggregation. Yet, direct evidence for such a scenario remains to be established. On the other hand, the mode of action of HSPB7 seems to be related to autophagic degradation as we found that it associates with misfolded proteins in a later stage, i.e. when they already have started to form oligomers. At this stage, HSPB7 seems to form a ring around the aggregates [14] (Fig. 2) by which it may prevent aggregate propagation such that the aggregates can be handled by the autophagy machinery. Furthermore, HSPB2 may play an even more intricate role, as neither HSP70s, autophagy or proteasome inhibition impaired its anti-aggregation activity (Fig. 3-4). Recent findings based on *in vitro* model showed that HSPB2 has protein-dependent chaperone activity and a significant ability to inhibit amyloid fibril formation of α -synuclein [43]. HSPB2 could represent an atypical chaperone by a unique, yet to be established mechanism.

In conclusion, the different HSPB members have both overlapping and divergent substrate specificities. The HSPBs support substrate refolding after acute stress, but also have degradation supportive activities in a disease mutant-specific manner. Thus, boosting HSPB expression or activity should be further explored as potential target for delaying folding diseases.

Materials & Methods

Plasmids and reagents. The human HSPB plasmid library used here was described before [14]. Flag-PARK2 WT and Flag-PARK2 C289G constructs were kindly provided by Prof. Michael E. Cheetham. Bortezomib (100 nM) was from Selleck Chemicals. 3-Methyladenine (3-MA, 10 mM), Wortmannin (200 nM) and VER155008 (40 μ M) were from Sigma.

Cell culture and transfection. Mouse embryonic fibroblasts (MEF), human embryonic kidney 293 (HEK293) cells expressing the tetracycline repressor (Flp-In T-Rex HEK293, Invitrogen Carlsbad, CA, USA) were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Gibco), Penicillin 100U/ml, and Streptomycin 100mg/ml (Gibco). Cells were grown at 37°C in 5% CO₂. HEK293 and MEF cells were transfected using Lipofectamine and Lipofectamine 2000, respectively (Invitrogen), according to the manufacturer's instructions using 1 mg of plasmid DNA per 35 mm dish. Gene expression was induced with 1 μ g/ml tetracycline. Cells were transfected for 24 hours with Flag-PARK2 C289G alone or together with the HSPBs (ratio 1:3). pcDNA3 or pcDNA5-FRTTO-mRFP were used as control conditions. Transfection of siRNA for HSPA1A (Dharmacon's SMARTpool siRNA) and a control sequence (Dharmacon's siCONTROL non-targeting siRNA) were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Immunofluorescence. HEK293 cells were plated on poly-D-Lysine (Sigma) coated cover slips. 24 hours after transfection, cells were washed twice with PBS (Gibco) and fixed with 2% formaldehyde (Sigma-Aldrich) for 10 minutes at room temperature. Cells were permeated using 0.1% Triton-X100 in PBS for 7 minutes at room temperature and later incubated in PBS+ (0,5% BSA and 0,15% Glycine in PBS). Cells were incubated with primary antibodies overnight at 4°C. Cells were washed four times with PBS+ and incubated with Alexa Fluor 488 Donkey Anti-Rabbit IgG (Invitrogen), Alexa Fluor 594 Donkey Anti-mouse IgG (Invitrogen) for 1.5 hours at room temperature, washed two times and mounted in glycerol (Agar Scientific). Confocal images were obtained using a confocal laser scanning microscope (Leica TCS SP8) with a 63X/1.32 oil objective. The % of aggregates in Flag-PARK2-positive cells was calculated.

Protein extraction and western blot. PARK2 C289G-aggregation was measured according to Rose et al. [21]. In short, transfected cells were washed with PBS and lysed in 1% Triton X-100 buffer, containing protease inhibitors (Roche), incubated in the lysis buffer for 15 minutes on ice and then separated by centrifugation at 14.000 RPM for 15 minutes at 4°C, to obtain Triton X-100 soluble (supernatant) and insoluble (pellet) fractions. Pellet fractions were re-suspended by sonication in Laemmli sample buffer (62.5 mM Tris-HCl buffer pH 6.8, 2% SDS, 10% glycerol, 10% b-mercaptoethanol and 0.001% bromophenol blue). Proteins were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and then processed for western blotting. Membranes were subsequently incubated with HRP-conjugated secondary antibodies (GE Healthcare) at 1:7000 dilution. Visualization was performed with enhanced chemiluminescence and Hyperfilm (ECL, GE Healthcare). Mouse monoclonal anti-FLAG (M2) was from Sigma, while mouse monoclonal anti-HSPA1A/Hsp70, anti-ubiquitin (FK2) and anti-HSPB1 were from Enzo LifeScience. Mouse monoclonal anti-PARK2 was from Cell Signalling Technology. Mouse monoclonal anti-GAPDH, anti-HSPB2, anti-HSPB4 and HSPB7 were from RDI Research Diagnostics, Transduction Laboratories, Abcam and Abnova, respectively. Rabbit polyclonal anti-FLAG and anti-H2A were from Abcam.

Statistical analysis. Results are expressed as mean \pm SEM. Statistical significance was analysed using an independent t-test. $P < 0.05$ was considered statistically significant, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$. All experiments were carried out at least three times, but only data of one representative experiment are shown.

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SUPPLEMENTARY DATA

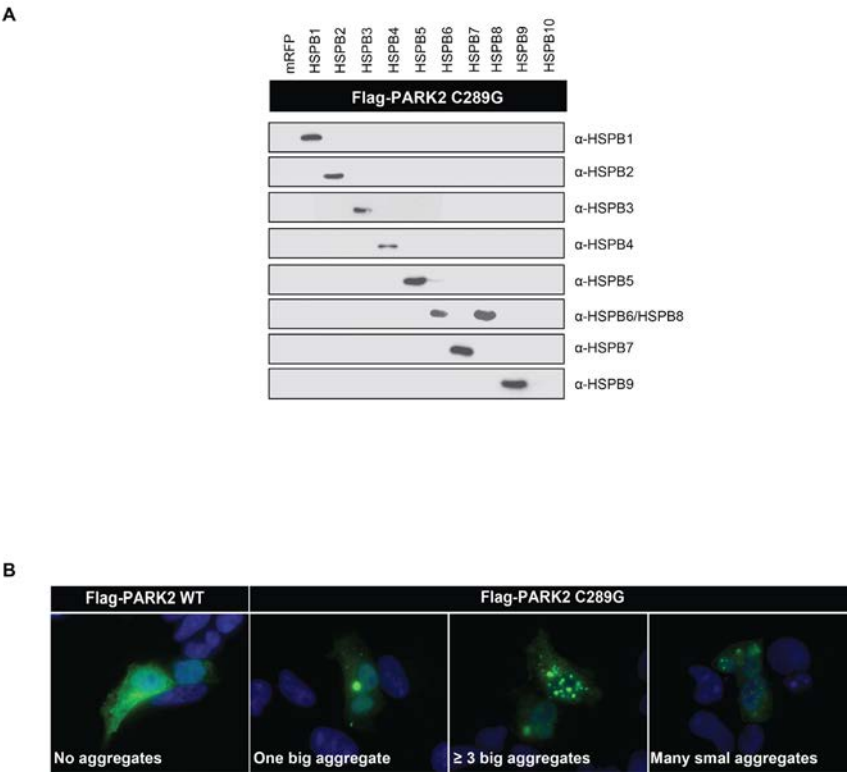


Figure S1. HSPBs expression levels and subdivision of PARK2 C289G-aggregates. (A) HSPB expression levels in HEK293 cells, transfected for 24hr with Flag-tagged PARK2 C289G alone or together with each of the different HSPB proteins. (B) Immunofluorescent staining of Flag-tagged PARK2 C289G (green) and dapi (blue) in HEK293 cells. Cells were divided in different categories.

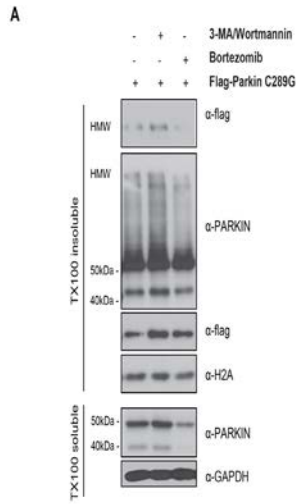


Figure S2. Role of ubiquitin proteasome system or the autophagy pathway in PARK2 C289G aggregates.

HEK293 cells were transfected with Flag-tagged PARK2 C289G and treated with Bortezomib (100 nM) or with 3-MA (20mM) and Wortmannin (200 nM) overnight. Cells were fractionated in TX-100 and analyzed for PARK2 C289G aggregates

